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Abstract

Mitogen Activated Protein Kinase (MAPK) pathways are ubiquitous among eukaryotes, and are involved in the transduction of various extracellular signals. In mammals, three MAPK pathways have been identified, two of these respond to stress, and one can stimulate growth and differentiation. MAPKs have also been found in plants, and it has been suggested that a MAPK pathway may be involved in the signal transduction of auxin and cytokinin, two plant hormones that stimulate growth and differentiation. However, at this time, there is no conclusive evidence supporting this hypothesis. By studying the effect of MAPKK's of both plant and animal origin in plant cells, we hope to establish a link between auxin signal transduction and the MAPK pathway.

Introduction

Mitogen Activated Protein Kinase, (MAPK), pathways are a conserved signaling motif found in organisms as evolutionarily distant as yeast and humans (reviewed by Seger and Krebs 1995). Different MAPK signaling pathways involved in the transduction of various extracellular signals in eukaryotic cells. Three critical proteins make up a MAPK pathway: MAPKKK, MAPKK and MAPK. Perception of the stimulus activates a MAPKKK, which phosphorylates and activates MAPKK, which in turn activates MAPK, which finally results in the phosphorylation of transcription factors that influence gene expression.

In mammals, three groups of MAPKs have been identified, the ERK, JNK and p38 MAP Kinases. Progress has been made in determining the factors leading to MAPK activation. For example, Phorbol ester and Epidermal Growth Factor, (EGF), activate ERK while only slightly activating p38 (Ahn *et.al.* 1992, Crews *et.al.* 1992). Conversely, UV radiation, osmotic stress and cytokines result in a significant increase in the activation of p38 (Raingeaud *et.al.* 1995). These results suggest that different pathways regulate the activation of different MAP Kinases. The ERK pathway is activated by signals associated with cell division and differentiation, while the p38 and JNK pathways are stress pathways (reviewed by Marshall 1995). MAPK Kinases can also be classified according to differences in structural features (Derijard *et.al.* 1995).

Four MAPK Kinases, the upstream activators of MAP Kinases, have also been found and their corresponding MAPK substrates identified. MEK1 and MEK2 activate ERK, MKK3 activates p38, and MKK4 activates both p38 and JNK (Derijard *et.al.* 1995).

Constituents of the MAPK pathway have also been identified in plants. Several studies have linked MAPK activation with stress response. Mechanical stimulation, wounding, cold and drought have all been shown to activate a MAP Kinase in higher plants (Bogre *et.al.* 1997, Jonak *et.al.* 1996, Seo *et.al.* 1995, Usami *et.al.* 1995). In *Arabidopsis thaliana*, MAPK and MAPKKK transcripts have been shown to be upregulated in response to drought, cold, touch and high salt concentrations (Mizoguchi *et.al.* 1996). Furthermore, elicitors of plant defense reactions activate MAP Kinases in tobacco (Adam *et.al.* 1997, Lebrum-Garcia *et.al.* 1998). Also similar to mammalian MAPKs, there has been some, evidence for MAPK involvement in plant cell division (Nakashima *et.al.* 1998).

Since auxin and cytokinin are two plant hormones able to stimulate plant cell division and differentiation, it has been suggested that MAPKs may play a role in their signal transduction (Mizoguchi *et.al.* 1994). However, as of yet, there is no conclusive evidence supporting this hypothesis. By studying the effect of MAPKK's of both plant and animal origin in plant cells, we hope to establish a link between auxin signal transduction and the MAPK pathway.

Results

Homology Between Plant and Animal MAPKKs

Five MAPKKs have been found in *Arabidopsis thaliana,* all sharing sequence homology (Fig 1). Of these five, AtMEK1 and AtMEK2 share the most sequence homology with the two human MAPK Kinases, MEK1 and MAPKK2, found to be involved in the MAP Kinase pathway for cell growth and differentiation (Table 1).

Table 1. Extent of Homology Among *Arabidopsis* and Human MAPKKs

	AtMEK1	AtMAPKK2	AtMAPKK3	AtMAPKK4	AtMAPKK5
HsMEK1	38(56)%	39(56)%	35(53)%	34(51)%	34(52)%
HsMAPKK2	37(56)%	37(56)%	35(53)%	34(50)%	32(49)%

Note – Number outside the bracket represents percent identity of amino acids, number inside the bracket represents percent similarity of the two protein sequences.

Furthermore, AtMEK1 is the only known MAPKK in *Arabidopsis thaliana* to contain two complementary serine residues, (S220:S224), to residues S218 and S222 in HsMEK1 that are phosphorylated upon activation (Fig 2). Therefore, it is likely that AtMEK1 is the plant version of HsMEK1, and is therefore the best candidate for growth and differentiation related auxin signal transduction pathway.

Effect of Constitutively Active HsMEK1 in Tobacco Cells

Since mammalian MEK1 is known to be involved in the proliferation and differentiation of animal cells (reviewed by Marshall 1995), and shares a high sequence homology to some plant MAPKKs, (Table 1 and Fig 2), mammalian MEK1 was used to study hormone signaling and the MAPK pathway in plants. Two versions of human HsMEK1 were obtained from Natalie Ahn's lab (Mansour *et.al.* 1994). The constitutively active form, HsMEK1+ (S218E:S222D), is 85 times more active than the original protein (Mansour *et.al.* 1994). The inactive form of MEK1,

HsMEK1- (K97M), was mutated at its ATP-binding site. This mutant can still interact with its upstream and downstream kinases, but it cannot activate MAPK. As a result, HsMEK1- is a dominant negative mutant.

In order to test the hypothesis that a MAPK pathway is involved in the regulation of cell proliferation and differentiation by plant hormones, HsMEK1+ was over-expressed in tobacco cells. Two days after HsMEK1 was introduced into tobacco cells through *Agrobacterium*-mediated transformation, the transformed cells and non-transformed cells were placed in medium with or without naphthalene acetic acid (NAA: synthetic auxin) or benzel amino purine (BAP: a synthetic cytokinin). Transformed and control cells produced microcalli after one week of growth in medium containing both hormones, indicating that cell division was occurring. However, in non-hormone medium, transformed cells began to form microcalli after one month, whereas control cells were dead by that time. The activated HsMEK1 turned on the proper hormone- signaling pathway in order to allow for cell growth and division in the absence of hormone. These results give evidence to the hypothesis that MAPKs are involved in hormone signal transduction.

Effects of Constitutively Active HsMEK1 on Transgenic Tobacco Plants

To further test our hypothesis, transgenic tobacco plants containing HsMEK1+ were made through *Agrobacterium*-mediated transformation. Five different kanamycin-resistant HsMEK1+ lines were obtained, each with a different level of expression of the mutant protein. The first generation plants were fertile, and did not display any unusual overall phenotypes. However, two of the lines recovered did display shorter roots than wild-type plants. At high

concentrations, auxin inhibits root elongation, and so these plants may have an over-sensitivity to auxin hormone. These two lines were used for further study.

The sensitivity of the transgenic plants to hormones was then tested. T1 and wild-type seeds were germinated in media containing various concentrations of auxin and cytokinin. The differences between wild-type and transgenic seedlings were most dramatic when the seeds were germinated in the dark in the presence of cytokinin. While wild-type seedlings had long roots, the HsMEK1+ transformants had short roots in 1uM cytokinin. At higher concentrations of cytokinin, (5uM), roots of transgenic seedlings turned into calli, and differentiated into shoots after being exposed to light. The formation of calli from the transgenic seedlings in the absence of auxin suggests that cells expressing HsMEK1+ had a higher sensitivity to auxin, since callus-formation in wild-type tobacco normally requires both auxin and cytokinin.

The capacity for cell division in the transgenic plants was monitored in order to further determine auxin sensitivity. Protoplasts were isolated from the leaves of T1 and wild-type tobacco plants. Within five days of protoplast isolation, both wild-type and transgenic protoplasts began to divide in the presence of both auxin and cytokinin. In the absence of both hormones, wild-type cells quickly died while HsMEK1+ cells still appeared healthy after one week. In the presence of cytokinin alone, HsMEK1+ transgenic cells were able to divide while wild-type cells were not. These results give additional evidence to the theory that the HsMEK1+ mutation leads to an increased sensitivity to auxin.

Effects of HsMEK1- on Transgenic Tobacco Plants

Using *Agrobacterium*-mediated transformation, transgenic tobacco plants over-expressing HsMEK1- were made. High levels of this protein were thought to block components of the MAPK pathway. Several overt phenotypes were observed in the transgenic plants, including the inability to form roots but form multiple apical meristems, asymmetrical flower petals, reduced number of petals, underdeveloped anthers, filaments fused to petals, and anther to petal transformation. Similar phenotypes have been previously reported in plants with increased cytokinin production, or in the presence of large quantities of exogenous cytokinin (Estruch *et.al.* 1993). In plants, auxin and cytokinin act as antagonistic hormones whose balance relative to the other is crucial for the proper effects of each. Since the HsMEK1- transgenic plants have a dominant-negative form of a MAPK, any pathway with MEK1 as one of its constituents will at least be partially blocked. Therefore, these HsMEK1- transgenic plants appear to have a decreased sensitivity to auxin due to a block in auxin signaling pathway by the mutant kinase. This would bias the perception of the two hormones in favor of cytokinin, resulting in the cytokinin-oversensitive phenotype observed.

Activation of Auxin-Inducible Promoter in Transgenic Protoplasts Expressing HsMEK1+

Protoplasts were isolated from HsMEK1+ and wild-type tobacco plants and then transfected with a GH3-GFP construct (Kavtun *et.al.* 1988). GH3 is an auxin-inducible promoter (Hagen *et.al.* 1991), while GFP is Green Fluorescence Protein. At a concentration of 0.1uM NAA, HsMEK1+ cells exhibited strong expression of GFP, while cells without the HsMEK1+ did not (Fig 3). These results indicate that HsMEK1+ increased the sensitivity of cells to auxin by activating the

auxin signal transduction pathway, thereby activating the auxin-inducible promoter driving GFP expression.

Effect of AtMEK1+ on Transgenic Tobacco and *Arabidopsis thaliana* Plants

As with HsMEK1, two mutants of AtMEK1 were created, one constitutively active, AtMEK1+ (S220E:S224D), and the other dominant-negative inactive, AtMEK1- (S220A:S224A). Using *Agrobacterium*-mediated transformation, AtMEK1+ transgenic tobacco and *Arabidopsis thaliana* plants were made. These plants have not yet matured, and so determining phenotypic abnormalities is not yet possible. We expect to see similar results to the experiments done using HsMEK1+, which includes transgenic plants with shorter roots, and the formation of calli in the absence of auxin from seedlings germinated in the dark at high cytokinin concentrations. We will test the sensitivity of these AtMEK1+ transformed cells to auxin using the GH3-GFP construct.

Effect of AtMEK1- on Transgenic Tobacco and *Arabidopsis thaliana* Plants

Using *Agrobacterium*-mediated transformation, AtMEK1- transgenic tobacco and *Arabidopsis thaliana* plants were made. These plants have also not yet matured, and so identifying aberrant phenotypes is still not possible. Again, we expect to find similar results to the experiments done using HsMEK1-, which includes transgenic plants with various phenotypic abnormalities characteristic of high cytokinin conditions.

Determination of the MAPK activated by AtMEK1

In order to characterize further the action of AtMEK1, we are in the process of attempting to determine which MAPK is activated by AtMEK1. In *Arabidopsis thaliana*, there are at least nine genes that encode MAPKs (Mizoguchi *et.al.* 1993, Mizoguchi *et.al.* 1997). Six of these, MAPK2-7, were placed under the control of a constitutive promoter, and tagged with hemagglutanin. Tobacco protoplasts were isolated and transfected with AtMEK1+ as well as one of the MAPKs obtained from *Arabidopsis thaliana.* Transfected protoplasts were allowed to sit for 12 hours, at which time protein extracts were obtained. Western immunoblotting was then performed using an antibody raised against the HA tag, to check for the expression of the proteins, and anti-activated-MAPK-Ab, to determine the activation state of the MAPKs. We have determined that the MAPK's can be expressed in the protoplasts, and we are still trying to determine the extent of AtMEK1+ expression. If one of the MAPKs used in the experiments is a substrate for AtMEK1, we expect to see a significant increase in its activation-state in the presence of AtMEK1+. The determination of this MAPK is important because, if the prior experiments are succesful, it is likely to be involved in the gene-activation terminating the auxin-signaling pathway.

Future Experiments

There are many other experiments that need to be performed in order to determine the connection between AtMEK1 and hormone signal transduction more clearly. Since auxin and cytokinin exhibit a characteristic dependence on each other, and seem to work antagonistically, it is likely that the signaling pathways of the two hormones share a great deal of interaction. It was shown that HsMEK1+ transgenic tobacco cells could still grow in the absence of hormones.

Both auxin and cytokinin are necessary for growth. Therefore, HsMEK1+ cells were able to compensate for the lack of auxin *and* cytokinin, which suggests that this MAPKK is involved in both auxin and cytokinin signal transduction. Therefore, it is important to try classify the role of AtMEK1 in cytokinin signal transduction.

In addition, different transgenic lines of tobacco and *Arabidopsis thaliana* express different amount of mutant protein. Depending on the amount of protein being expressed, different transgenic plants may have different phenotypes. Therefore, it is important to classify the phenotypes with the amount of protein being expressed.

Methods

Mutants of AtMEK1

AtMEK1 has been previously cloned and the sequence deposited into GenBank (Morris *et.al.* 1997, Acession # - AAB97145). The primers used to clone AtMEK1 were as follows:

5'-CCA GcC ATG gAC AGA GGA AGC TTA TG-3'

5'-Gag gCc tGT TAG CAA GTG GGG GAA TC-3'

Lower case letters indicate regions where mutations were introduced in order to establish restriction sites to facilitate later genetic manipulation. An NcoI site (CCATGG) was introduced in the beginning of the gene, and a StuI site (AGGCCT) was established at the end of the gene. Using PCR-mediated site directed mutagenesis , AtMEK1+ (S220E:S224D) and AtMEK1- (S220A:S224A) were made. The overlapping primers used to incorporate the proper mutations were as follows:

S220E-S224D primers:

5'-ATG TGC CCA CGA AAt cAT TAG CAA Gct cAC TTG TGC TTG TC-3'

5'-GAC AAG CAC AAG Tga gCT TGC TAA Tga TTT CGT GGG CAC AT-3'

S220A-S224A primers:

5'-ATG TGC CCA CGA AAg cAT TAG CAA GAg cAC TTG TGC TTG TC-3'

5'-GAC AAG CAC AAG Tgc TCT TGC TAA Tgc TTT CGT GGG CAC AT-3'

(Lower case letters indicate regions where mutations were introduced)

Results were sent to a sequencing facility for verification of proper mutations.

Transgenic Plants

A Construct including 35SC4PPDK promoter (Sheen 1993, EMBO-12), HA-tagged mutant MEK1, and *nos* terminator was inserted into the pART27 binary vector (Gleave 1992). The

resulting plasmid was introduced into *Agrobacterium tumefaciens* EHA105 through electroporation. In tobacco, *Nicotiana tabacum* SR1 leaves were transformed (Chiu 1996), and kanamycin-resistant plants were selected. *Agrobacterium*-mediated transformation of *Arabidopdis thaliana* was performed using the floral dip method (Clough and Bent 1998).

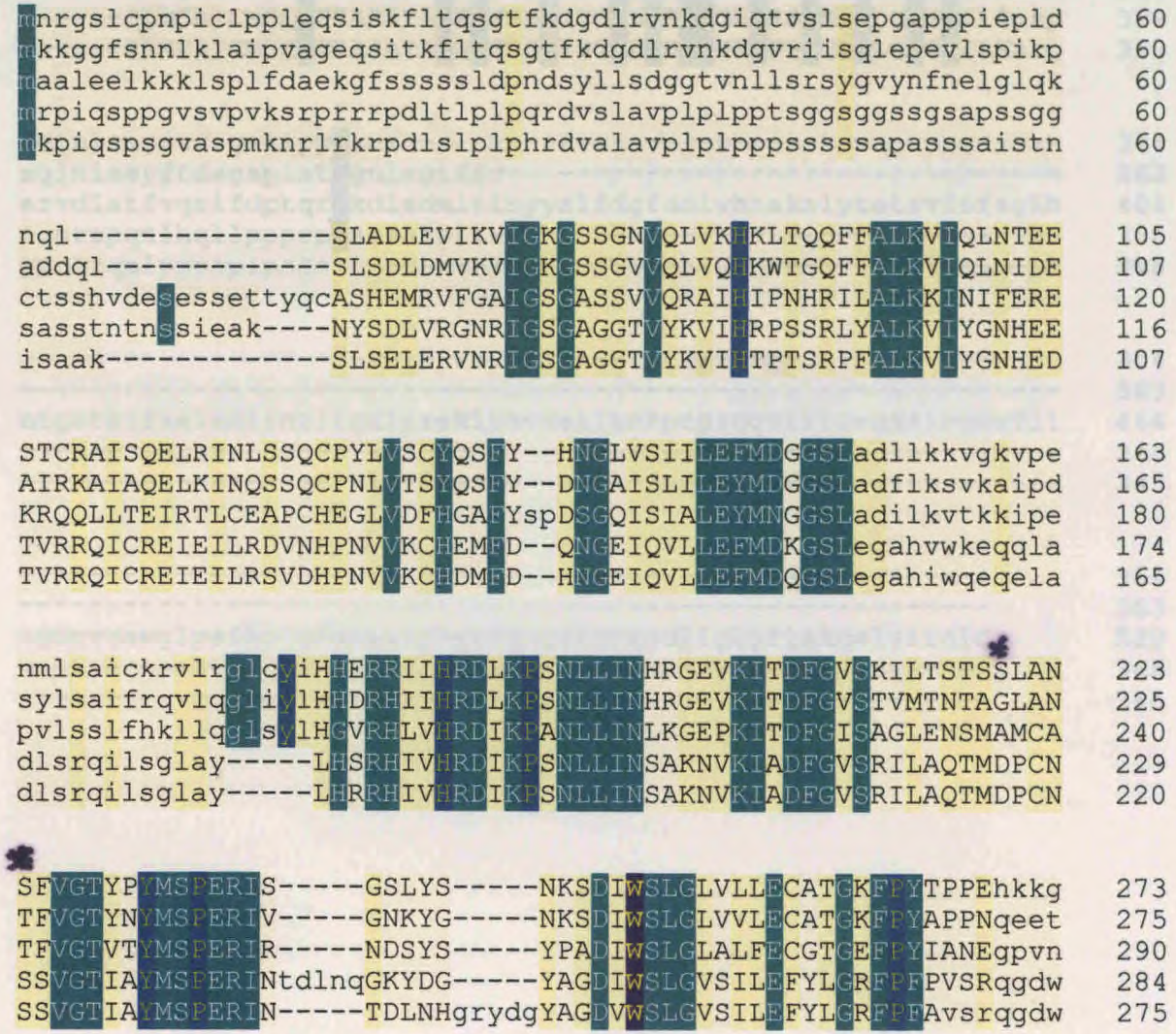
Protoplast Transient Expression

Healthy and expanded tobacco SR1 leaves were cut to about $2cm^2$ and digested in an enzyme solution consisting of 1.2% Cellulase R10 and 0.4% Macerozyme R10 in K3 medium, (Nagy 1976), with 0.4M sucrose. Cells were left overnight in the dark at 23°C. Floating protoplasts were selected. Plasmid DNA was added (10ug) to 0.25ml of freshly isolated tobacco protoplast cells (10^6 ml^{-1}) in 0.4M mannitol, 20mM $CaCl_2$, 5mM MES, pH 5.7. An equal volume of 40% PEG in 0.4M mannitol and 100uM $Ca(NO_3)_2$ (brought to pH 10 using KOH before autoclaving) was added immediately, mexed well and incubated for 10 minutes at room temperature. The mix was diluted with 4ml of K3 medium containing 0.3M sucrose. The transfected protoplasts were incubated in the dark for 20-24 hours before being photographed.

MEKs

Alignment

Figure 1



MEKs

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AtMEK1      wssvyeLVDAIVENPPPCAPSNLFSPEFCSFISQCVQKDFRDRKSAKELLEHKFVKMfed   333
AtMAPKK2    wtsvfeLMEAIVDQPPPALPSGNFSPELSSFISTCLQKDFNSRSSAKELMEHPFLNKydy   335
AtMAPKK3    ------LMLQILDDPSPTPPKQEFSPEFCSFIDACLQKDFDARPTADQLLSHPFITKhek   344
AtMAPKK4    a-----SLMCAICMSQPPEAPATASPEFRHFISCCLQREPGKRRSAMQLLQHPFILRasp   339
AtMAPKK5    a-----SLMCAICMSQPPEAPATASQEFRHFVSCCLQSDPPKRWSAQQLLQHPFILKatg   330

AtMEK1      sdtnlsayftdagslippllan----------------------------------------   354
AtMAPKK2    sginlasyftdagsplatlgnlsgtfsv-------------------------------   363
AtMAPKK3    ervdlatfvqsifdptqrlkdladmltihyyslfdgfddlwhhakslytetsvfsfsgkh   404
AtMAPKK4    sqnrspqnlhqllppprplsssssptt---------------------------------   366
AtMAPKK5    gpnlrqmlppprplpsas------------------------------------------   348

AtMEK1      ----------------------------------------------------------   354
AtMAPKK2    ----------------------------------------------------------   363
AtMAPKK3    ntgsteifsalsdirntltgdlpseklvhvveklhckpcgsggviiravgsfivgnqfli   464
AtMAPKK4    ----------------------------------------------------------   366
AtMAPKK5    ----------------------------------------------------------   348

AtMEK1      ----------------------------------------------------        354
AtMAPKK2    ----------------------------------------------------        363
AtMAPKK3    cgdgvqaeglpsfkdlgfdvasrrvgrfqeqfvvesgdligkyflakqelyitnld    520
AtMAPKK4    ----------------------------------------------------        366
AtMAPKK5    ----------------------------------------------------        348
```

Alignment Figure 2

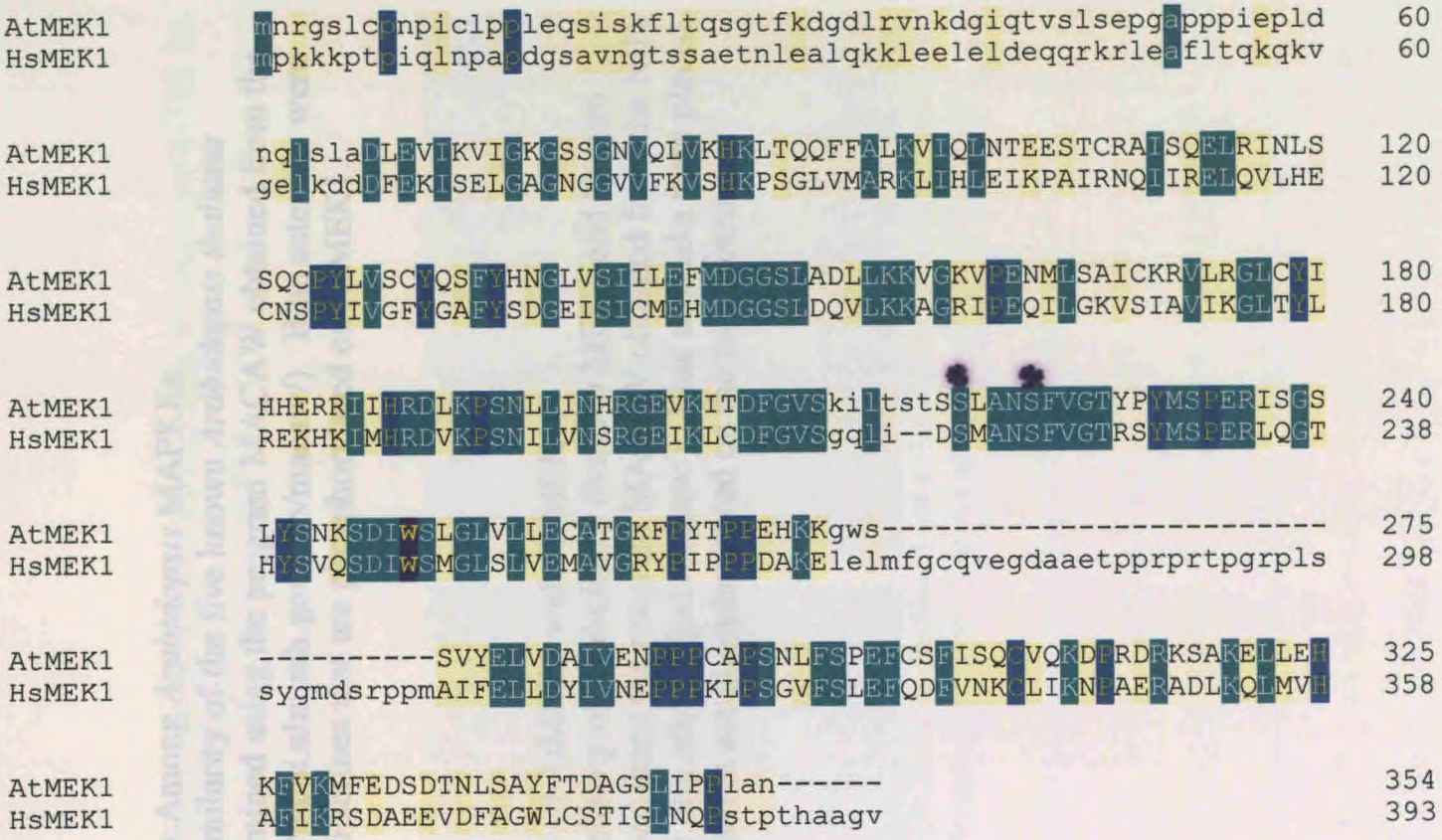

Figure 1. Homology Among *Arabidopsis* MAPKKs.
Protein sequences similarity of the five known *Arabidopsis thaliana* MAPKKs was determined using the program MACAW obtained from the NIH homepage (ftp://ncbi.nlm.nih.gov/pub/macaw/). Blue asterisks were placed above the two serines that are phosphorylated on AtMEK1.

Figure 2. Homology of AtMEK1 with HsMEK1.
Protein sequence similarity of *Arabidopsis thaliana* MEK1 and Human MEK1 was determined using the program MACAW obtained from the NIH homepage (ftp://ncbi.nlm.nih.gov/pub/macaw/). Blue asterisks were placed above the two serines that are phosphorylated on the two proteins.

Figure 3. Activation of the Auxin-Inducible Promoter GH3 in HsMEK1+ Cells

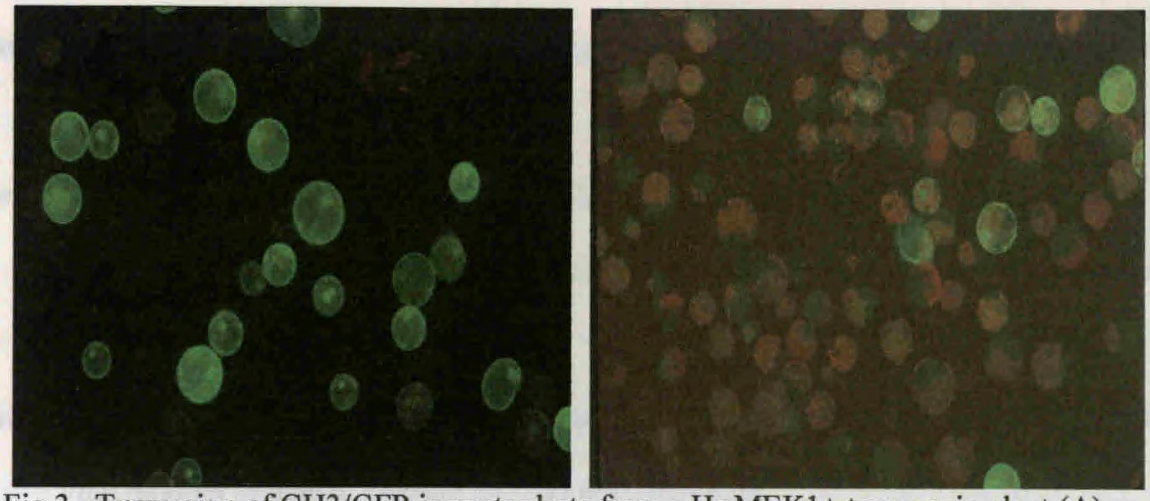

Fig 3. Txpression of GH3/GFP in protoplasts from a Hs MEK1+ transgenic plant (A) or a wild-type plant (B). Pictures were taken 12 hours after induction by 0.1uM NAA.

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